A peer-reviewed open-access journal
NeoBiota
Advancing research on alien species and biological invasions

NeoBiota 101: 243–275 (2025)
DOI: 10.3897/neobiota.101.153292

Research Article

Ponto-Caspian amphipods (Crustacea, Amphipoda) and their microsporidian parasites recently established in Sweden

Tomasz Rewicz[1], Andrea Desiderato[1], Jennie Barthel Svedén[2], Karolina Bacela-Spychalska[1], Tomasz Mamos[1], Ulf Bjelke[3]

1 *Department of Invertebrate Zoology and Hydrobiology, University of Lodz, Banacha str., 12/16, 90-237, Łódź, Poland*
2 *County Administrative Board of Stockholm, Box 22067, SE-104 22 Stockholm, Sweden*
3 *Swedish Species Information Centre, Swedish University of Agricultural Sciences, Almas allé 8E, Box 7016, SE-750 07 Uppsala, Sweden*
Corresponding author: Tomasz Rewicz (tomasz.rewicz@biol.uni.lodz.pl)

Abstract

The invasive amphipods *Dikerogammarus villosus*, *D. haemobaphes* and *Pontogammarus robustoides*, which originate from the Ponto-Caspian region, are amongst Europe's most ecologically disruptive amphipods and have recently been detected in Sweden, marking their northernmost record and indicating a concerning expansion of their range. This study employed DNA barcoding to confirm the species identifications, assess population structure and trace the origins of these recently introduced populations. To improve the detection resolution in the case of *D. villosus*, we designed specific primers targeting two additional mitochondrial markers at genes *ND5* and *ATP8*, enabling precise identification of the probable source populations, based on known European invasion routes and their phylogeographic structure. Our analysis identified the Western Group of *D. villosus* as being present in Sweden, with a probable introduction from the large lakes in the foothills of the Alps, possibly via sailing boats that were transported overland. Meanwhile, *P. robustoides* and *D. haemobaphes* appear to have invaded from the eastern and southern Baltic regions — including the Baltic States, Vistula Lagoon and Szczecin Lagoon — possibly via ballast water, ship hulls or floating debris. Additionally, we detected microsporidian infections in all three species, identifying *Dictyocoela muelleri* and *D. berillonum* as associated parasites. Given the ecological risks posed by these invasive species, coordinated monitoring, particularly along the Baltic coast, is essential for enabling rapid response efforts in the Nordic region.

Key words: *Dikerogammarus haemobaphes*, *Dikerogammarus villosus*, mitochondrial marker, *Pontogammarus robustoides*, range expansion, source of introduction

OPEN ACCESS

Academic editor: Adam Petrusek
Received: 18 March 2025
Accepted: 24 August 2025
Published: 2 October 2025

Citation: Rewicz T, Desiderato A, Svedén JB, Bacela-Spychalska K, Mamos T, Bjelke U (2025) Ponto-Caspian amphipods (Crustacea, Amphipoda) and their microsporidian parasites recently established in Sweden. NeoBiota 101: 243–275. https://doi.org/10.3897/neobiota.101.153292

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Introduction

Freshwater ecosystems are invaluable, harbouring about 6% of global species despite covering only 0.01% of Earth's water resources and 0.80% of its surface (Strayer and Dudgeon 2010). They play important environmental, economic, cultural and scientific roles and are aesthetically valuable elements of the landscape, which is fundamental for tourism (Dudgeon et al. 2006; Hall and Härkönen 2006; Harrison et al. 2010). However, these vital ecosystems are severely threatened, surpassing the biodiversity loss of terrestrial habitats (Hadwen et al. 2012; Venohr et al. 2018). Reports show that only 40% of EU waters meet satisfactory ecological status (European Environmental Agency 2018). Inland waters, particularly lakes,

are highly susceptible to biological invasions driven by various human activities (Gherardi 2007; Ricciardi and MacIsaac 2011). Commercial shipping facilitates the introduction of alien species primarily through biofouling (Lacoursière-Roussel et al. 2016) and ballast waters (Briski et al. 2012), while recreational activities like angling (Carpio et al. 2019), boating (Podwysocki et al. 2024), canoeing (Anderson et al. 2014) and diving, also contribute to the introduction and spread of invasive species (Bacela-Spychalska et al. 2013). Additionally, intentional releases further exacerbate the situation (Keller et al. 2009), posing a significant threat to the ecological balance of lakes worldwide.

Invasive alien species receive considerable attention in Scandinavia and Northern Europe. In that region, freshwater ecosystems are relatively young, having developed over the past 10,000–15,000 years, following the retreat of the last ice sheet (Berglund 1979). The Nordic region is a global hotspot for lakes, along with northern North America and parts of Russia (Messager et al. 2016). Approximately 9% of Sweden's territory consists of freshwaters, with 97,500 lakes and 298,000 smaller waterbodies (0.2–1 ha) (Henriksen et al. 1998; Verpoorter et al. 2012). However, glaciation has stripped soils, leaving exposed bedrock and resulting in generally low nutrient and calcium levels in many waterbodies, which may explain the absence or rarity of benthic crustaceans, especially Amphipoda, in calcium-poor regions of Sweden (Sahlén Zetterberg and Fölster 2022). According to Sweden's NOBANIS database (Nobanis 2024), 2,497 alien species, including 453 arthropods, have been recorded in the country. Amongst these are a few dozen freshwater aquatic arthropod species recorded in lakes and other watercourses. Four invasive amphipods: *Chelicorophium curvispinum* (Leppänen et al. 2017), *Dikerogammarus haemobaphes*, *D. villosus* and *Pontogammarus robustoides* (Artfakta Sweden 2025) have been detected prior to 2025.

Amphipods may be harmful invasive species due to their potential to outnumber native invertebrate species, leading to a decline in native biodiversity (Jazdzewski et al. 2004; Piscart et al. 2010; Dick et al. 2013). Additionally, tube-building amphipods can alter habitat structures and ecosystem processes, causing significant ecological changes in invaded areas (Rigolet et al. 2014). Amongst invasive amphipods, those originating from the Ponto-Caspian region (Azov, Black, Caspian and Aral seas and adjacent area) pose a significant threat to inland European waters (Bij de Vaate et al. 2002; Grabowski et al. 2007; Soto et al. 2023); up to 40% of those have spread outside their native range during the last century (Copilaș-Ciocianu et al. 2023a). Ponto-Caspian species, which have evolved in environments with frequent fluctuations of salinities, water level and temperature, possess unique biological features favourable for survival under unstable conditions, making them successful invaders (Cristescu et al. 2003; Cristescu and Hebert 2005; Copilaș-Ciocianu and Sidorov 2022; Copilaș-Ciocianu et al. 2023a). Their invasive spread, facilitated by such anthropogenic activities as increased shipping, boating, canal construction and intentional introductions, has led to the establishment of vital populations and, subsequently, competitive superiority over native species, resulting in declines and extinctions along invasion corridors (Leuven et al. 2009). They have used three main European migratory corridors (Bij de Vaate et al. 2002), transferring genetically distinct populations from isolated areas in their native range to new territories.

Amongst invasive Ponto-Caspian amphipods, *D. villosus*, *D. haemobaphes* and *P. robustoides* are the most successful invaders, which have colonised the majority of the main inland waterbodies of Europe within less than 30 years (Rewicz et al.

2014; Jażdżewska et al. 2020; Copilaș-Ciocianu et al. 2023a, 2023b), exerting a detrimental impact on local benthic communities. *Dikerogammarus* spp. have even earned the nicknames 'killer shrimp' or 'demon shrimp' due to their severe impacts on local fauna, making them some of the most threatening invasive invertebrates in freshwater ecosystems (Bacela-Spychalska and Van der Velde 2013; Rewicz et al. 2014; Warren et al. 2021). *Pontogammarus robustoides* is also a large (up to 21 mm) invasive amphipod that significantly impacts the native species communities, occupying a relatively high trophic position due to its mixed diet of animal prey and plant detritus (Bacela-Spychalska and Van der Velde 2013).

All three species are known to be the hosts of a variety of microsporidian parasites (Ovcharenko et al. 2010; Bacela-Spychalska et al. 2012, 2018; Bojko et al. 2019; Warren et al. 2023), which are dangerous hitchhikers during the invasion process and are amongst the most common ones out of the diversity of recognised micro- and macro-parasites in Amphipoda (Bojko and Ovcharenko 2019; Bojko et al. 2022; Bacela-Spychalska et al. 2023). These highly reduced unicellular parasites (e.g. lacking mitochondria and possessing small ribosomes) infect both vertebrate and invertebrate species (Vávra and Lukeš 2013). Their transmission modes can be vertical, horizontal or both (Stentiford and Dunn 2014), with diverse impacts on host populations, from decreasing the predatory pressure of the host on native communities (horizontally transmitted) (Bacela-Spychalska et al. 2012) to increasing the potential for fast host population growth by, for instance, feminising the host (some vertically transmitted) (Slothouber Galbreath et al. 2004). Finally, if infected host amphipods get introduced into a new area, they may serve as a new threat to native fauna and serve as vector for the parasites to local species.

Molecular methods, such as DNA barcoding and environmental DNA (eDNA) analyses, provide precise tools for the accurate identification and detection of freshwater invasive species, including various amphipod taxa (Blackman et al. 2017; Thomas et al. 2020; Copilaş-Ciocianu et al. 2022). The markers most often used in barcoding, such as mitochondrial genes for the cytochrome c oxidase subunit I (COI) and 16S rRNA in the case of animals, often show intraspecific variability levels which allow population-scale analysis. For instance, the genetic variation of mtDNA markers observed in *D. haemobaphes* and *P. robustoides* was high enough to make it possible to infer their colonisation history (Jażdżewska et al. 2020; Copilaş-Ciocianu et al. 2023b). On the other hand, intraspecific variation in mtDNA markers of *D. villosus* is low and does not allow for population studies for the determination of colonisation pathways, as has been shown by Rewicz et al. (2015a).

Recent studies have provided mitochondrial genomes (Mamos et al. 2021; Macher et al. 2023), which can be used as source data for the identification of highly variable regions and developing molecular markers that show variability on an intraspecific level. Such new markers, in combination with eDNA-based biomonitoring (Bommerlund et al. 2023), may provide important information about the origin and dispersal of invasive species (Andres et al. 2023a, 2023b). Phylogeographic and population genetics data for all three species have been obtained across their invaded and native ranges (Rewicz et al. 2015a, 2017; Jażdżewska et al. 2020; Copilaş-Ciocianu et al. 2022, 2023b). In the case of *D. villosus*, the two distinct invasive populations, one derived from the Danube and the other from the Dnieper, have been detected in Central and Western Europe. These populations exhibit genetic differentiation and are allopatrically distributed, with no indication of diminished genetic diversity relative to their respective source regions (Rewicz et al. 2015a, 2017).

Designing a reliable molecular tool for the rapid detection of a specific group of *D. villosus* could provide a better understanding of their invasiveness (Hupało et al. 2018; Podwysocki et al. 2024, 2025b; Jermacz et al. 2025).

This study aims to: a) use DNA barcoding to assess the genetic diversity of the recently established invasive amphipods *D. villosus*, *D. haemobaphes* and *P. robustoides* in Sweden; b) design primers for variable mitochondrial markers to determine the source populations of the killer shrimp *D. villosus*; c) investigate whether populations of *D. villosus*, *D. haemobaphes* and *P. robustoides* are infected by microsporidian parasites, which are common hitchhikers during invasion and assess their potential impact on the host invasion process.

Materials and methods

Sampling in Sweden

The littoral invertebrate fauna of Swedish lakes and streams was surveyed through environmental monitoring programmes and the three species included in this study were discovered via these surveys and shared via online repositories. The initial discovery of *D. villosus* in Sweden (Bjelke and Tomasson 2023) was made in 2023, based on material collected in 2022. *Dikerogammarus haemobaphes* was first identified in 2023, but a re-examination of older samples revealed its presence at one locality as early as 2017 (Artfakta Sweden 2025). *Pontogammarus robustoides* was recorded for the first time in 2023 (Artfakta Sweden 2025).

Freshly sampled material of gammarids for molecular studies was obtained through kick-sampling with a benthic hand-net. Subsequently, the amphipods were preserved in 96% ethanol and later identified in the laboratory to the species level. Individuals of *D. villosus* were collected in Lake Vättern (58.290°N, 14.643°E) on 24 February 2023 by Ulf Bjelke. Individuals of *D. haemobaphes* were collected in Lake Tullingesjön, close to Stockholm (59.221°N, 17.877°E) on 28 April 2023 by Jennie Barthel Svedén and Joakim Pansar. Individuals of *P. robustoides* were collected in Lake Mälaren, close to Stockholm (59.504°N, 17.789°E) on 28 October 2023 by Ulf Bjelke.

Additional sampling in Europe

To test for the source populations of these individuals, the additional samples of the three species coming from native and invaded range collected during different sampling campaigns spanning from 2013 to 2019 (Fig. 1, Suppl. materials 1, 2) were used. All the samples are stored in the Department of Invertebrate Zoology and Hydrobiology, University of Lodz.

DNA extraction, DNA barcoding

Samples were processed either in the Canadian Centre for DNA Barcoding (CCDB), Guelph, Canada or in the Department of Invertebrate Zoology and Hydrobiology, University of Lodz (UniLodz) (see Suppl. material 2 for each specimen details). DNA extraction was done at UniLodz according to the method of Bacela-Spychalska et al. (2018), to ensure that microsporidian DNA was co-extracted properly with the host DNA. Amplification of standard

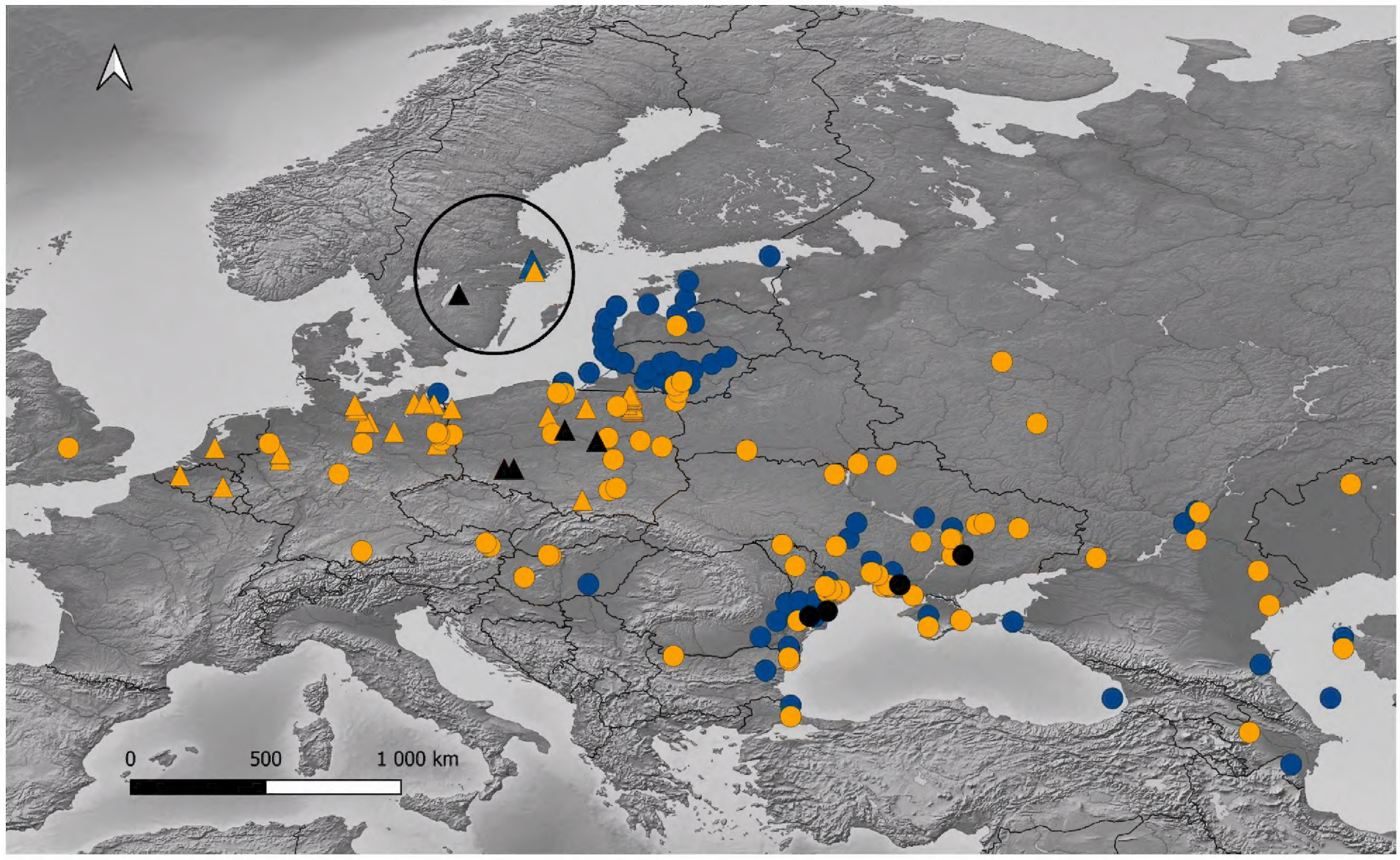

Figure 1. Geographic distribution of sequenced individuals analysed in this study. New data (triangles) and public database sequences (circles) are shown for *Dikerogammarus villosus* (black), *D. haemobaphes* (yellow) and *Pontogammarus robustoides* (blue). The locations of the Swedish records used in this study are encircled. Further details can be found from Suppl. material 1.

animal DNA barcode gene region (COI) (Hebert et al. 2003) and sequencing were done following Morhun et al. (2022).

The Barcode Index Number (BIN; Ratnasingham and Hebert (2013)) method, part of the Barcode of Life Data Systems (BOLD; Ratnasingham and Hebert (2007)), was used for molecular species identification through DNA barcoding. Newly-submitted sequences were compared with those already available in BOLD. Sequences were clustered on the basis of molecular divergence using algorithms designed to detect discontinuities between the clusters. Each cluster was assigned a unique BIN, either matching an existing BIN or a newly-created one if the submitted sequences did not align with known BINs. All BINs are registered in BOLD.

Selecting the new markers and primers designed for *Dikerogammarus villosus*

As Rewicz et al. (2015b) have pointed out, the COI marker is not variable enough to distinguish the molecular groups of *D. villosus* in Europe. To avoid a complicated and costly procedure involving the testing of microsatellite markers, two new markers for *D. villosus* were prepared. To design them, the most SNP-rich mitogenome coding fragments identified by Mamos et al. (2021) were targeted when comparing mitogenomes of *D. villosus* from Poland, England and Turkiye (populations from both the Eastern and Western Groups sensu Rewicze et al. 2015 and an isolated group from the native region). The Primer 3 software was used to design new primers for the amplification of the new markers (Untergasser et al. 2012).

The first marker is located in the gene coding for the NADH-ubiquinone oxidoreductase chain five protein (ND5, 5047 bp to 5990 bp of the complete mitochondrial genome, GenBank accession number: OK173836). To amplify it, newly-designed primers: ND5DvF (forward; 5'-GATGTAGCTATTTTATTA-AGG-3') and ND5DvR (reverse; 5'-TTCCTCCCAACAATGTCAC-3') were used. The second marker (position 1994 bp to 2864 bp of OK173836) encompassed fragments of the cytochrome c oxidase subunit II (COX2, 397 bp), full sequences of genes for tRNA-Lys, tRNA-Asp, an ATP synthase membrane subunit 8 (ATP8) and partial sequence of the gene coding ATP synthase membrane subunit 6 (ATP6; 307 bp). For the amplification of this region, the newlydesigned primers: C2ATPDvF (forward; 5'-CCTTTTGGAGACTGATAA-CAG-3'), C2ATPDvR (reverse; 5'-CAAGTGAGAGTGTAAAAATTAAC-3') were used. The PCR conditions were as follows: an initial denaturing step at 94 °C for 3 min was followed by 35 cycles of 94 °C for 20 s, 47 °C for 45 s and 72 °C for 1 min, with a final extension at 72 °C for 2 min. Primers were checked for their specificity for *D. villosus* against other gammarids, using mitogenomes available from Mamos et al. (2021) *in silico* and empirically through the PCR reaction with samples of *D. villosus*, *D. haemobaphes*, *D. bispinosus*, *Obesogammarus crassus* and *Pontogammarus robustoides*, as other prominent invasive Ponto-Caspian amphipods occurring together with the target species in sympatry in the invaded range in Europe (Morhun et al. 2022; Macher et al. 2023).

Data assembly and analysis

The newly-obtained sequences of amphipods were edited, aligned with MUSCLE (Edgar 2004) and trimmed using Geneious v.10.2.6 (Kearse et al. 2012) and deposited in GenBank (COI: PP390747–PP391012, PV135977–PV136006; ATP8: PP420950–PP421043; ND5: PP390565–PP390658) and BOLD (Ratnasingham and Hebert 2007). Additional COI sequences with relevant voucher information of *D. haemobaphes* (Cristescu and Hebert 2005; Grabner et al. 2015; Sket and Hou 2018; Jażdżewska et al. 2020; Copilaş-Ciocianu et al. 2022, 2023b; Morhun et al. 2022) and *P. robustoides* (Cristescu et al. 2003; Cristescu and Hebert 2005; Hou et al. 2011, 2014; Sket and Hou 2018; Csabai et al. 2020; Copilaş-Ciocianu et al. 2022, 2023b) that are publicly accessible through the public dataset DS-SWEAMP (https://doi.org/10.5883/DS-SWEAMP) on the BOLD (www.boldsystems.org) were used.

The haplotype relationship was visualised through Median-Joining Networks (MJN) using PopART (Leigh and Bryant 2015). Networks for *D. villosus* were constructed from different datasets: 94 COI sequences (605 bp), as well as for 94 ND5 and ATP8 sequences (855 bp, 758 bp, 1613 bp concatenated) and, in the last approach, 94 sequences of ND5, ATP8 and COI (2,218 bp concatenated). Network for *D. haemobaphes* was constructed based on 643 COI sequences (543 bp) and network for *P. robustoides* was constructed based on 201 COI sequences (574 bp). Individuals of *D. villosus* were *a priori* assigned to the invasion routes, according to Rewicz et al. (2015a). Samples from the Danube and Dnieper deltas presented in Suppl. material 1 represent the native ranges of the species, reflecting the origin of the Western and Eastern Groups. Samples from the Oder and the Vistula reflect the endpoints of invasions of those groups so far, as presented by Rewicz et al. (2015a) and together they should encompass the majority of the known molecular diversity of those populations. For the main lineage

of *D. haemobaphes*, we assigned individuals to the invasion route, according to Jażdżewska et al. (2020). We indicated other lineages (not invasive so far), based on information from Jażdżewska et al. (2020) and Copilaş-Ciocianu et al. (2022). *Pontogammarus robustoides* lacks any comprehensive phylogeographical studies, so we divided it into major regional groups, based on the recommendations of Csabai et al. (2020) and Copilaş-Ciocianu et al. (2023b).

The genetic diversity of the studied markers and taxa was evaluated by determining haplotype count (k) and haplotypic diversity (h), following the methodology proposed by Nei (1987). The calculations were performed using DnaSP v.5 software (Librado and Rozas 2009).

To evaluate the differences between the studied molecular markers and between the two invasion routes (comprising the native range) of *D. villosus* and the Swedish population, an analysis of molecular variance (AMOVA) was conducted with 10,100 permutations on each gene with Arlequin v.3.5.2.2 (Excoffier and Lischer 2010). Three groups were tested, the Eastern, the Western and Sweden, for a total of nine populations (i.e. four per route + Sweden). The analyses were conducted using both a strictly orthogonal design, considering only the groups and a nested one, including the differences between individual populations sampled within each group. Both approaches were used to provide a more robust analysis and comprehensive understanding of the molecular variance. The F-statistics were calculated from haplotype frequencies using 10,100 permutations to assess the significance of the pairwise comparisons.

Microsporidia screening

Specimens of *D. villosus*, *D. haemobaphes*, and *P. robustoides* from Sweden were tested for the presence of microsporidian intracellular parasites. Microsporidian DNA was co-extracted together with the host DNA and amplification of microsporidian partial 16S rDNA was performed via PCR using microsporidia — specific primers V1f (forward) and 530r (reverse) (Vossbrinck et al. 1993; Baker et al. 1994). Detailed protocols describing reaction conditions and agarose gel checking followed Kobak et al. (2021). All infected individuals were selected for further microsporidian SSU rDNA, ITS and partial LSU rDNA operon amplification for more accurate taxonomic identification. We used the microsporidia — specific primer pair V1f and 580r (reverse) (Weiss et al. 1994). In some cases, when we could not obtain sufficiently long fragments in one reaction, we amplified them as the two overlapping amplicons using primer pairs: Vif with MC3r (Ovcharenko et al. 2010) and Hg4f (Gatehouse and Malone 1998) with 580r (details presented by Bacela-Spychalska et al. (2018)). In cases when the amplification of the long fragment was unsuccessful, we sequenced the initial short fragment, which is adequate for general parasite species determination (Quiles et al. 2020).

Microsporidia identification

The obtained sequences were checked with a BLAST search (Altschul et al. 1990). As all our sequences were identified as *Dictyocoela* spp., 434 other available *Dictyocoela* sequences from GenBank with a minimum length of 500 bp and four other microsporidian species as an outgroup were added to our dataset sequences (Suppl. material 3), based on published studies (Nilsen et al. 1998; Hogg

et al. 2002; Terry et al. 2004; Lovy et al. 2009; Ovcharenko et al. 2010; Bojko et al. 2015; Stentiford et al. 2017; Bacela-Spychalska et al. 2018; Park et al. 2020). This dataset was used to illustrate the relationship between our sequences and as many available *Dictyocoela* spp. sequences in GenBank as possible, with the use of Bayesian Inference. Sequences were aligned using MAFFT7 software (Katoh and Standley 2013) with the E-IONS-I algorithm using the legacy gap penalty option, incorporated in Geneious v.10.2.2 (Kearse et al. 2012). To improve the tree resolution and species identification, we refined our dataset by removing all short sequences and leaving an alignment of SSU rDNA, ITS and partial LSU rDNA (sequence length 970–1,767 bp, final alignment length 1,930 bp) and single representatives of haplotypes. Both phylogenetic reconstructions were performed using Bayesian Inference implemented in MrBayes plugin v. 2.2.4 (Huelsenbeck and Ronquist 2001) integrated in Geneious v.10.2.2. The best-fitting model of nucleotide substitution was determined with ModelTest-NG v.0.1.7 (Darriba et al. 2020). We used the General Time Reversible (GTR) model with gamma-distributed rate heterogeneity (+G) and the proportion of invariable sites (+I). The MCMC was set to 1,100,000 iterations with 100,000 burn-in and four heated chains, sampled every 200 iterations. Runs parameters reached satisfactory effective sampling sizes (ESS > 100). Fifty percent majority-rule consensus trees were constructed. Newly-obtained sequences of microsporidian SSU, ITS and partial LSU rDNA fragments were edited, aligned and trimmed using Geneious v.10.2.6 (Kearse et al. 2012) and deposited in GenBank under accession numbers PP074278–PP074300 and PV170728–PV170732.

Results

Sampling campaigns and monitoring

Extensive sampling campaigns, conducted by the local authorities after the initial findings of *D. villosus* in Lake Vättern, showed its presence at 26 sites (Bjelke and Tomasson 2023; Bjelke 2024). This invasive species was a dominant community member in nine of them. Forty-two sites, including the whole northern and eastern banks of the lake, remained uncolonised by *D. villosus* (Fig. 2). *Dikerogammarus haemobaphes* has so far been detected at two sites in Lake Mälaren. Additional occurrences have been recorded in Lakes Albysjön and Tullingesjön, which are close to Lake Mälaren and drain into it. In contrast, *P. robustoides*, also discovered in 2023, appears to be spreading more rapidly in Lake Mälaren, having been recorded from at least eight sites already (Bjelke 2024).

Molecular identification of Amphipoda

We used a COI mtDNA barcoding marker and confirmed the morphological identification of *D. villosus.* Thirty-two individuals collected in Sweden were assigned to BIN BOLD:AAI9938, which contains all known European individuals of *D. villosus*. Twenty-six individuals of *D. haemobaphes* were assigned to BIN BOLD:AEO1330, the most widespread and invasive lineage occurring in Europe. All 30 individuals of *P. robustoides* were assigned to BIN BOLD:AAB7665, which is currently the only known BIN grouping COI sequences of this species.

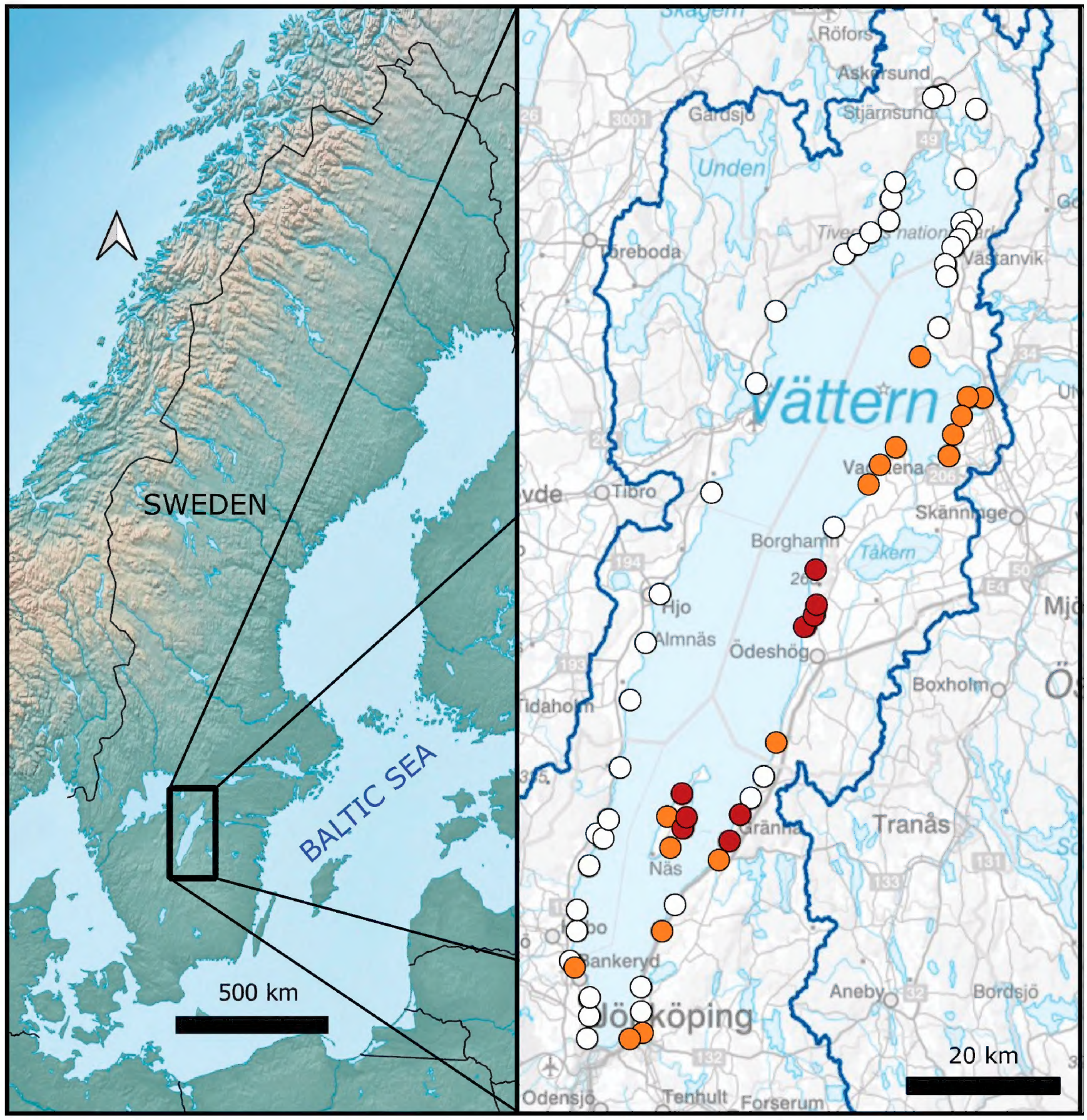

Figure 2. Results of *D. villosus* sampling in the Lake Vättern area by the local environmental agency in 2024. The colour of the dots represents the stage of invasion: white — no records; orange — a few individuals, but not the dominant species; red — dominant species.

Newly-designed primers for ATP8 and ND5 markers were amplified from all individuals from Sweden and also for selected reference samples from Poland and Ukraine (Suppl. material 1). The primers exhibited full specificity for *D. villosus* and did not amplify any closely-related species. We showed that COI alone could not discriminate between Western and Eastern Groups as the most common haplotype overlaps between the routes (Table 1, Suppl. material 6). The newly-designed ND5 and ATP8 markers individually revealed only a single haplotype shared between specimens from both the Western and Eastern Groups, their concatenation providing greater resolution. This combined dataset produced unique haplotype combinations that allowed us to determinate the geographic origin of *D. villosus* individuals, as shown in the Median Joining Network (Fig. 3A). The specimens from Sweden were assigned to two haplotypes; one corresponded to a haplotype detected in the Western Route (Oder, Danube), the other was a private haplotype differentiated by one base pair. Adding COI to the dataset further increased the

Table 1. Comparison of the discriminatory power of newly-designed markers for the molecular groups, based on 94 *D. villosus* reference sequences from the native and invaded range.

	length [bp]	variable sites	Hd	H
COI	605	6	0.250	1/6
ND5	855	16	0.612	1/11
ATP8	758	13	0.752	1/10
ND5+ATP8	1613	29	0.785	0/16
ND5+ATP8+COI	2218	35	0.790	0/18

Note: Hd — haplotypic diversity; H — The number of haplotypes shared by both Eastern and Western Groups, followed by the total number of haplotypes.

resolution, as two additional haplotypes (one from Oder – Western Group, one from Vistula – Eastern Group) were detected, but the general pattern (Suppl. material 6) remained unchanged, as was the case with ND5+ATP8.

The analysis of molecular variance indicated distinct patterns of spatial variation across the mitochondrial genes examined (COI, ATP8 and ND5; Table 2).The orthogonal AMOVA for the COI gene showed that most of genetic variation (88.3%) was observed within groups, with significant, but low differentiation amongst groups (11.7%, F_{ST} = 0.117, $p < 0.003$, see Table 2 for detailed results). Furthermore, the hierarchical AMOVA for the COI gene revealed a complex population structure. A negative and non-significant F_{CT} value (–0.061, $p > 0.05$) suggested little differentiation amongst the broader groups. Instead, the majority of the variation was found amongst populations within groups, with a significant F_{SC} of 0.333 ($p < 0.005$) and an overall within-population F_{ST} of 0.293 ($p < 0.001$). In contrast to the COI gene, both ATP8 and ND5 exhibited substantial genetic differentiation amongst groups. For ATP8, 45.5% of the total genetic variation was attributed to differences amongst groups, as shown by both the orthogonal (F_{ST} = 0.565, $p < 0.001$) and hierarchical (F_{CT} = 0.478, $p < 0.003$) analyses. This pattern was even more pronounced for ND5, which displayed an even higher level of differentiation with 65.5% of variation amongst groups (orthogonal F_{ST} = 0.725, $p < 0.001$; hierarchical F_{CT} = 0.669, $p < 0.03$). For the hierarchical AMOVA of the ATP8, the variation amongst populations within groups was 17.3% (F_{SC} = 0.332, $p < 0.001$) with an overall within-population F_{ST} of 0.651 ($p < 0.001$). Similarly, for ND5, variation amongst populations within groups was 11.1% (F_{SC} = 0.336, $p < 0.002$) with an overall within-population F_{ST} of 0.780 ($p < 0.001$).

The pairwise F_{ST} values differed markedly amongst the studied genes. While F_{ST} values for ATP8 were consistently high (> 0.77) in all comparisons with the Swedish population, the patterns for ND5 were more variable. Specifically, a high F_{ST} of ~ 0.90 was observed between the Swedish and Eastern Groups, whereas the value was considerably lower (~ 0.30) for the Western Group (Suppl. material 5). In contrast, pairwise F_{ST} for COI showed no difference between the Swedish population and the two populations from both of the Western and Eastern Groups, due to shared dominant haplotype amongst all these groups.

Our dataset for *D. haemobaphes* consisted of 649 COI sequences (179 newly generated in this study; see Suppl. materials 1, 2 for details). Visualisation of the haplotype relationship showed us the main European invasive lineage (BOLD:AEO1330) as dominant in Sweden and the rest of the invaded range. Additionally, there are three distinct lineages of Caspian Sea origin (BOLD:AAX9262, BOLD:AEO1331,

Table 2. Results of the analyses of molecular variance (AMOVA). The results marked in bold are significant (p-value < 0.05) after 10,100 permutations. Gene: mitochondrial DNA marker; Source of variation: how genetic diversity is partitioned (Amongst groups, Amongst populations within groups, Within populations); d.f.: degrees of freedom; Sum of squares: measure of total variation; Variance components: estimated genetic variance attributable to each source; Percentage of variation: proportion of total genetic variation explained by each source; Fixation Index: F-statistics (F_{ST}: overall genetic differentiation; F_{CT}: differentiation amongst groups; F_{SC}: differentiation amongst populations within groups).

Gene	Source of variation	d.f.	Sum of squares	Variance components	Percentage of variation	P-value	Fixation Index
COI	Amongst groups	2	1.154	0.015	**11.7**	**0.002**	**F_{ST}: 0.117**
	Within groups	91	10.495	0.115	**88.3**		
	Total	93	11.649	0.131			
	Amongst groups	2	1.154	-0.008	-6.1	0.572	F_{CT}: -0.061
	Amongst populations within groups	6	2.661	0.046	**35.4**	**0.005**	**F_{SC}: 0.333**
	Within populations	85	7.834	0.092	**70.8**	**0.000**	**F_{ST}: 0.293**
	Total	93	11.649	0.130			
ATP8	Amongst groups	2	16.418	0.264	**56.5**	**0.000**	**F_{ST}: 0.565**
	Within groups	91	18.561	0.204	**43.5**		
	Total	93	34.979	0.468			
	Amongst groups	2	16.418	0.224	**47.8**	**0.003**	**F_{CT}: 0.478**
	Amongst populations within groups	6	4.687	0.081	**17.3**	**0.000**	**F_{SC}: 0.332**
	Within populations	85	13.874	0.163	**34.9**	**0.000**	**F_{ST}: 0.651**
	Total	93	34.979	0.468			
ND5	Amongst groups	2	18.209	0.297	**72.5**	**0.000**	**F_{ST}: 0.725**
	Within groups	91	10.249	0.113	**27.5**		
	Total	93	28.457	0.410			
	Amongst groups	2	18.209	0.274	**66.9**	**0.030**	**F_{CT}: 0.669**
	Amongst populations within groups	6	2.606	0.046	**11.1**	**0.002**	**F_{SC}: 0.336**
	Within populations	85	7.643	0.090	**22.0**	**0.000**	**F_{ST}: 0.780**
	Total	93	28.457	0.409			

BOLD:AEO3554), detected in Kazakhstan, Azerbaijan and Russia (Jażdżewska et al. 2020; Copilaş-Ciocianu et al. 2022) and a cryptic lineage BOLD:ADB9467 occurring in the native area, in lower Danube and Dniester (Fig. 3B). Individuals from Sweden were assigned to two haplotypes: the dominant one was shared with the specimens from both Southern and Central corridors (25 individuals), one Swedish individual carrying a haplotype otherwise common in Ukraine and Poland (Fig. 3B).

To reveal the potential source population for *Pontogammarus robustoides* in Sweden, we used the COI dataset consisting of 201 sequences (30 from Sweden, see Suppl. material 1 for details). Swedish individuals were assigned to two haplotypes. Twenty-nine individuals were assigned to a haplotype already detected in the Baltic Region and the Dnieper and one individual was assigned to a second, newly-identified haplotype clustering with others from the same area (Fig. 3C).

Microsporidia screening

Amongst 32 individuals of *D. villosus*, 26 individuals of *D. haemobaphes* and 30 individuals of *P. robustoides* that were screened for microsporidia presence, we detected one, 25 and five infected individuals, respectively (Suppl. material 2). The prevalence of the confirmed infections was 3% for *D. villosus*, 96% for *D. haemobaphes* and 17% for *P. robustoides*. A single specimen of *D. villosus* was infected by *Dictyocoela muelleri*.

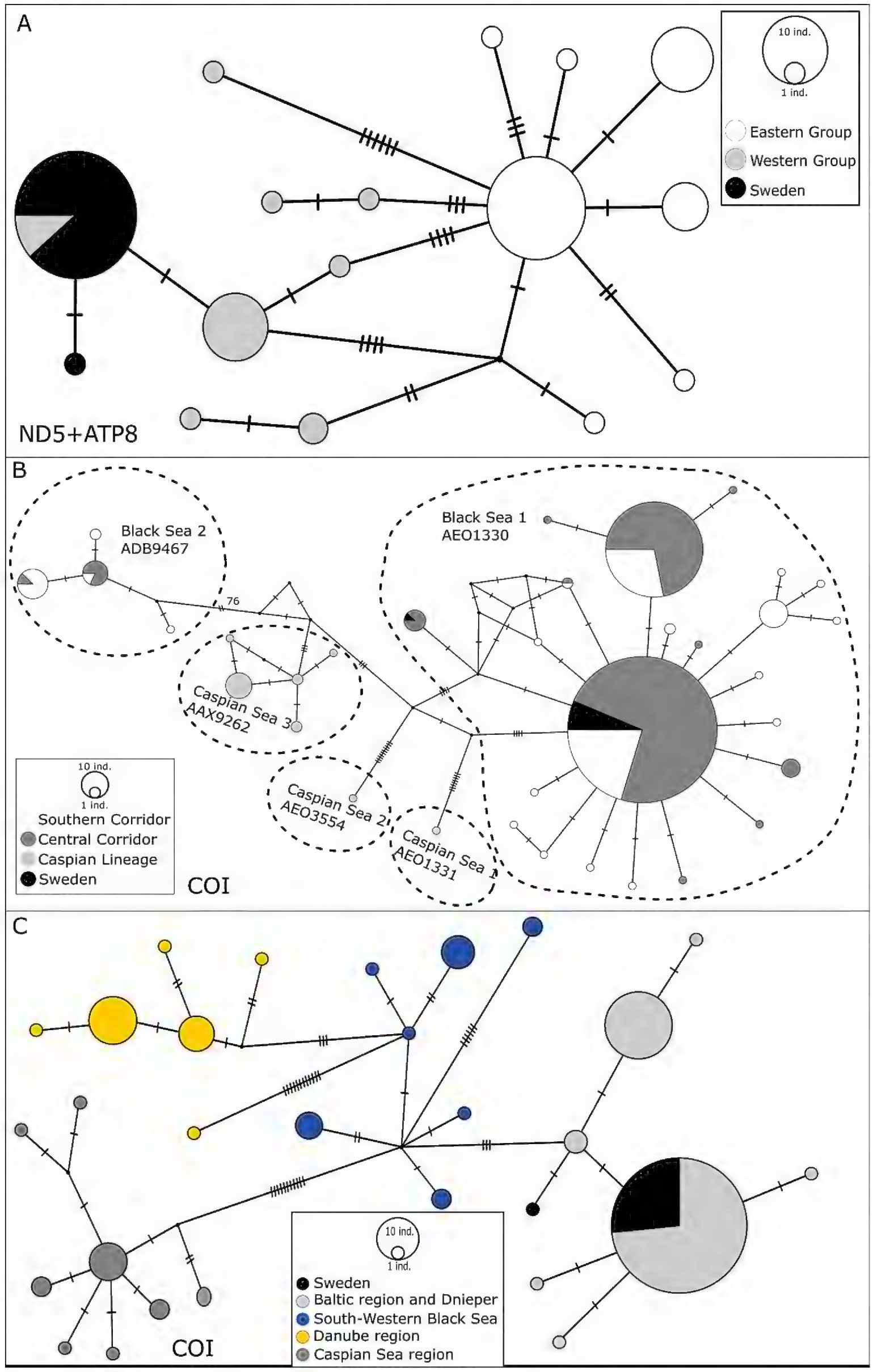

Figure 3. Median-joining haplotype networks showing relationships between haplotypes for the three studied amphipod species. **A.** *Dikerogammarus villosus*, based on concatenated ATP8 and ND5 dataset; **B.** *Dikerogammarus haemobaphes*, based on COI dataset; **C.** *Pontogammarus robustoides*, based on COI dataset. Each bar represents a single mutational step, with small black dots indicating undetected intermediate haplotype states. Circle sizes are proportionate to haplotype frequencies, as illustrated by the open circles with accompanying numbers. In all panels, colours represent individuals from different geographic regions. Dashed lines in B encircle distinct Barcode Index Numbers (BINs) indicated by their respective codes.

Dikerogammarus haemobaphes was also infected by *D. muelleri*, which was detected in 16 individuals and *D. berillonum* infected seven individuals of this host species. Five individuals of *P. robustoides* were infected by *D. berillonum.* In the case of three potentially infected individuals of *D. haemobaphes*, we had a PCR product of the desired length, but we could not successfully sequence even a short fragment. Thus, we presume a microsporidia infection as well.

Fourteen of the *D. muelleri* sequences obtained in this study were identical to those already found in *D. haemobaphes*, but from its native range in Ukraine (Bacela-Spychalska et al. 2018). This haplotype, together with the other newly-produced sequences, are grouped within the *D. muelleri* clade, characteristic of the Ponto-Caspian hosts (Fig. 4) (Bacela-Spychalska et al. 2018). A single infected individual of *D. villosus* carried the same parasite haplotype as *D. haemobaphes*. All seven individuals of *D. haemobaphes* were infected by the same haplotype of *D. berillonum. Pontogammarus robustoides* individuals were infected by two haplotypes of *D. berillonum* (three and two individuals, respectively), which were newly identified, but grouped with other haplotypes specific to Ponto-Caspian hosts (Fig. 4).

Discussion

Invasive Ponto-Caspian amphipods are steadily spreading into new water-bodies and watersheds across Europe (Copilaş-Ciocianu and Šidagytė-Copilas 2022; Podwysocki et al. 2024), making them some of the most successfully invasive aquatic species in the region. These amphipods and other Ponto-Caspian crustaceans and mussels dominate the macroinvertebrate communities in major European rivers, a phenomenon called "faunal Ponto-Caspianisation" (Soto et al. 2023). Recently, three prominent species — *Dikerogammarus villosus*, *D. haemobaphes* and *Pontogammarus robustoides* — have been found in Sweden, carrying parasitic microsporidia, which has heightened concern for the integrity of Scandinavia's freshwater ecosystems. Here, we discuss possible introduction pathways, population structures and the use of newly-developed molecular tools to assist in the monitoring of these invasive species. These findings open the door for developing a rapid, group-specific, eDNA-based monitoring kit specifically for *D. villosus*.

Population structure and possible introduction pathways of the new invaders

Dikerogammarus villosus, commonly known as the "killer shrimp", is expanding its range in Europe and has recently successfully colonised the Masurian Lakes in Poland (Podwysocki et al. 2024) and the coasts of Lithuania and Latvia in the Eastern Baltic Sea (Copilaș-Ciocianu et al. 2023a). The population discovered in 2022 in Lake Vättern, with its molecularly confirmed identification in this study from samples collected in 2023, represents the first recorded occurrence of *D. villosus* from the Scandinavian Peninsula.

DNA barcoding of the Swedish population revealed the presence of only a single haplotype, the most common one found across Europe (Suppl. material 6). Lack of higher genetic diversity suggests that the introduction probably occurred through a single event involving a limited number of individuals. Previous phylogeographic studies by Rewicz et al. (2015a, 2017) demonstrated that neither COI alone nor its combination with 16S are sufficient to determine the origin of invasive

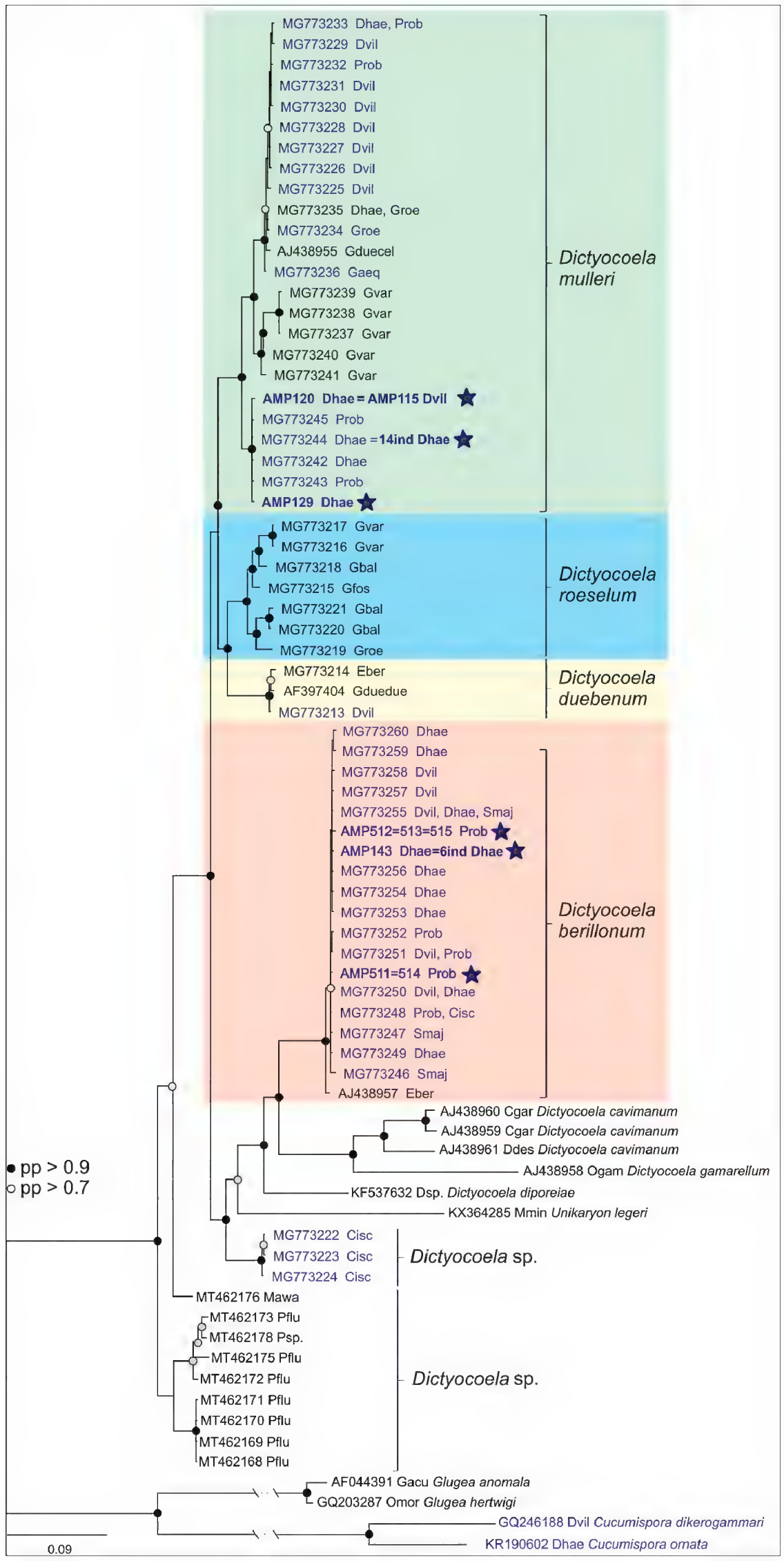

Figure 4. Bayesian phylogenetic reconstruction of *Dictyocoela* spp., based on fragments containing SSU, ITS and partial LSU genes. For parasite sequences, the labels include the GenBank accession number and the abbreviation of the host species name (see below). Sequences representing haplotypes from this study are indicated in bold and with a star; sequences from the Ponto-Caspian hosts are indicated in blue. pp: Bayesian Posterior Probability. Host species: Cgar – *Cryptorchestia garbini*, Cisc – *Chaetogammarus ischnus*, Ddes – *Deshayesorchestia deshayesii* (= *Talorchestia deshayesi*), Dhae – *Dikerogammarus haemobaphes*, Dvil – *Dikerogammarus villosus*, Dsp. – *Diporeia* sp., Eber – *Echinogammarus berilloni*, Gaeq – *Gammarus aequicauda*, Gbal – *Gammarus balcanicus*, Gduedue – *Gammarus duebeni duebeni*, Gduecel – *Gammarus duebeni celticus*, Gfos – *Gammarus fossarum*, Groe – *Gammarus roeselli*, Gvar – *Gammarus varsoviensis*, Mawa – *Melita awa*, Mmin – *Marteilia minutus*, Ogam – *Orchestia gammarellus*, Omor – *Osmerus mordax*, Pflu – *Paracalliope fluviatilis*, Psp. – Paraleptamphopidae sp., Prob – *Pontogammarus robustoides*, Smaj – *Spirogammarus major*.

populations of *D. villosus*. Instead, specific microsatellite markers have successfully distinguished the phylogeography of the species, identifying the Dnieper as the source of the Eastern Group and the Danube as the source of the Western Group. This study presents an alternative to time-consuming and costly microsatellite markers by using two newly-designed ND5 and ATP8 mitochondrial markers. By testing these markers on both native and invasive populations from the Eastern and Western Groups, we demonstrated their usefulness and discriminatory power for assessing the origin of the invasive populations (Table 1, Fig. 3A). Concatenated fragments revealed distinct haplotypes for each group with no overlap between them. Using individuals from the native range previously analysed by Rewicz et al. (2015a), we confirmed their assignment to major groups. Our findings confirm that individuals invading Sweden originated from the Western Group (Fig. 3A).

The most probable introduction pathway for *D. villosus* into Lake Vättern is via fishing boats transported from Lake Constance in recent years (U. Bjelke, personal communication). Extensive sampling conducted in 2023 (Fig. 2) revealed the presence of *D. villosus* primarily on the Lake's eastern shore, which is more accessible to tourists and equipped with boat ramps. This scenario is plausible given the species' well-documented resistance to desiccation and ability to attach to ropes, hulls and lines (Bacela-Spychalska et al. 2013). The presence of the Western Group in subalpine lakes and particularly Lake Constance, further supports this hypothesis (Rewicz et al. 2017). *Dikerogammarus villosus* is known for its ability to successfully colonise lakes, as demonstrated in the lakes of the Alpine foothills and Masurian Lakeland (Bacela-Spychalska et al. 2013; Rewicz et al. 2017; Podwysocki et al. 2024). In both cases, leisure activities enhancing the probability of the species' introduction were identified as the most probable vectors for its dispersal. A similar scenario probably facilitated the colonisation of the British Isles, where further studies implicated tourists and anglers as primary vectors (MacNeil et al. 2010; Anderson et al. 2015; Smith et al. 2020). Lake Vättern is connected to the Baltic Sea via the Göta Canal; however, sampling surveys in at least three sites on this canal did not reveal *D. villosus* (Suppl. material 7). On the other hand, we cannot rule out a reverse scenario in the future, where individuals of the killer shrimp may colonise the canal and continue eastwards to reach the Baltic coast and move north into the Stockholm area. The Göta Canal also continues westwards to Lake Vänern and through the Trollhätte Canal to the Kattegat in Göteborg. Monitoring the whole watershed seems crucial to track this invasion dynamic and directions.

Dikerogammarus haemobaphes, a prominent Ponto-Caspian invader, has expanded its range along the Eastern Baltic coast in recent years, with a newly-recorded location in Riga Bay (Copilaş-Ciocianu and Šidagytė-Copilas 2022). A recent synthesis of Ponto-Caspian amphipod distribution in Europe (Copilaș-Ciocianu et al. 2023a) identifies this site as the northernmost record within the Baltic Sea. Populations discovered in Lake Mälaren and Lakes Albysjön and Tullingesjön, near Stockholm, represent a significant extension of the species' range by over 400 km and across the Baltic Sea, marking an important advance in its invasive spread northwards.

DNA barcoding and phylogeographic studies of *D. haemobaphes* (Jażdżewska et al. 2020; Copilaş-Ciocianu et al. 2022; Copilaş-Ciocianu and Šidagytė-Copilas 2022), primarily based on the COI marker, provided a comprehensive overview of the population structure and genetic diversity of *D. haemobaphes* in newly-established populations and partially also in its native range. Our study shows that the Swedish population does not originate from the three Caspian Sea lineages or the lineage from

the Danube and north-western part of the Black Sea as revealed by Jażdżewska et al. (2020), none of which are invasive in mainland Europe so far and all of them are cryptic regarding their morphological features. Furthermore, haplotypes discovered in Sweden do not correspond to haplotypes specific to the Southern Corridor sensu Bij de Vaate et al. 2002 (Fig. 3B). Instead, the most probable sources of the introduction to the Stockholm area appear to be either from the east or south of the Baltic, including Riga Bay, Vistula Lagoon, Gulf of Gdańsk, Szczecin Lagoon or Rügen. These areas harbour *D. haemobaphes* populations (Jazdzewski et al. 2004; Dobrzycka-Krahel et al. 2016; Meßner and Zettler 2018; Copilaș-Ciocianu et al. 2023a) capable of tolerating brackish water conditions. Haplotypes discovered in the Swedish population seem to be the most common ones present in the southern Baltic Region.

The high dispersal capacities of invasive Ponto-Caspian amphipods have facilitated their successful colonisation of Europe (Copilaș-Ciocianu et al. 2023a) and the North American Great Lakes (Witt et al. 1997). The most plausible mechanisms for translocating *D. haemobaphes* across the Baltic Sea include transport in the ballast water of commercial vessels or attachment to smaller tourist vessels. This species has previously demonstrated its capacity to cross sea barriers, having reached Great Britain in 2012 (Aldridge 2013; Constable and Birkby 2016). The low salinity of the Baltic Sea offers a favourable environment for *D. haemobaphes*, eliminating physiological barriers to its spread. Moreover, the Baltic Sea is a busy maritime region with extensive commercial and recreational traffic and Stockholm maintains direct connections with Gdańsk and several Baltic and German ports, which may constitute source areas for introductions (Liebuvienė and Čižiūnienė 2021; Baran and Neumann 2023). Experimental studies by Dobrzycka-Krahel and Graca (2014) indicate that *D. haemobaphes* prefers salinities of around 7 PSU (though it is also known to be a successful invader in freshwater environments). Such a value matches the salinity levels in the Stockholm region and the Gulf of Riga, which are approximately 7 PSU in open seawater and much lower, even down to freshwater level, in gulfs and estuaries (Jaspers et al. 2021; Pinseel et al. 2022). This enhances the probability of establishment of the new populations. Furthermore, the attachment ability of this species to ropes and rough surfaces is superior even to other Ponto-Caspian species and greatly outperforms native gammarids (Bacela-Spychalska 2016).

Pontogammarus robustoides, another successful invasive amphipod of Ponto-Caspian origin, has expanded its European range in recent years, with new records from Estonia (Copilaş-Ciocianu and Šidagytė-Copilas 2022), Hungary (Csabai et al. 2020) and the Netherlands (Moedt and van Haaren 2018). Similar to *D. haemobaphes*, its discovery in the Lake Mälaren area in 2023 represented a substantial range extension and the first occurrence across the Baltic Sea.

Despite its widespread invasion, a comprehensive phylogeographic study of *P. robustoides* in its non-native range is still lacking. Studies of mitochondrial COI data from Csabai et al. (2020) confirmed that the Hungarian population originates from the Danube Basin. Copilaş-Ciocianu et al. (2023b) compared native and Baltic populations, supporting the hypothesis that this species was introduced directly to the Baltic States. Initially, *P. robustoides* became acclimatised in the Kaunas Water Reservoir (WR), Lithuania (Jażdżewski 1980; Vaitonis et al. 1990; Arbačiauskas et al. 2017). From there, it was either deliberately introduced into other waterbodies and neighbouring countries prior to the late 1980s or naturally

dispersed throughout much of the Baltic Basin and beyond (Arbačiauskas et al. 2011; Moedt and van Haaren 2018; Meßner and Zettler 2021). Primary sources of these translocations were the newly-built Dnieper and Simferopol water reservoirs in Ukraine, which were stocked with specimens from the Dnieper-Bug Estuary where the species is native (Gasiunas 1972; Arbačiauskas et al. 2017).

Swedish individuals were assigned to two haplotypes: one dominant in the Baltic Region and Dnieper and a second, newly-identified haplotype clustering with others from the same area (Fig. 3C). This finding supports the most probable scenario — similar to that of *D. haemobaphes* — that *P. robustoides* was translocated to the Stockholm area from the south-eastern Baltic Region, such as the Baltic States, Vistula Lagoon and Szczecin Lagoon. *Pontogammarus robustoides* seems to have spread much faster than *D. haemobaphes* in the Lake Mälaren area. Although it was first detected six years later than the latter species (2023 to 2017), it has already been reported from many more localities and its spread is more dynamic (Suppl. materials 4, 7).

Importance of tracking invaders at the population level

Traditionally, research on invasive species has focused on species-level characteristics to identify traits associated with invasiveness and predict potentially invasive species (Peoples and Goforth 2017). However, recent studies have shifted towards a population-level perspective, emphasising the dynamics of individual populations within invasive species (Haubrock et al. 2024; Sousa et al. 2024). This shift is appropriate in the case of *D. villosus*, where Rewicz et al. (2015a) highlighted genetic variability within source populations and isolation between Eastern and Western Groups in Europe, which may significantly influence their invasion success. Subsequently, Hupało et al. (2018) have demonstrated differing metabolic responses in Heat Shock Proteins (HSPs), showing that the Eastern Group invests more resources into cellular defence under thermal stress, potentially increasing its environmental tolerance and thus increasing its potential for invasiveness. Podwysocki et al. (2025a; 2025b; in press) have revealed differences in food preferences, dispersal capacity and morphology between these two *D. villosus* molecular groups. These findings underscore the importance of rapidly tracking the origin of invasive species populations. Our newly-designed markers offer a promising solution with the potential for population-specific monitoring using environmental DNA (eDNA) and previously collected specimens for the rapid differentiation of the two groups.

Additionally, an admixture of the Western and Eastern Groups is possible, forming intraspecific hybrids. This scenario has been found to be plausible in Poland, where a gap of no more than 50 km wide in the Notecki Canal separates the two groups (T. Rewicz, A. Desiderato, unpublished data). Given the connectivity of waterbodies and the invader dispersal abilities in Sweden, similar scenarios cannot be excluded. The Eastern Group currently occupies coastal areas of the south-eastern Baltic (Copilaş-Ciocianu and Šidagytė-Copilas 2022), potentially following a route similar to *D. haemobaphes* in its spread to Scandinavia. Jermacz et al. (2025) tested the metabolic response of both *D. villosus* groups and their hybrid to temperature increases and oxygen deficiency. Results suggest that, while large individuals and the Western Group are more sensitive to environmental stressors, intraspecific hybridisation may enhance the invaders'

environmental tolerance. These findings align with observations by Brauer et al. (2023) and Kulmuni et al. (2024), who reported that hybridisation facilitates adaptation to rapid environmental changes, reducing vulnerability to climate change. Hybridisation may also enhance genetic variation within populations and contribute to hybrid vigour, as Facon et al. (2005) have suggested.

Microsporidia hitchhikers and implications for the freshwater biodiversity in Sweden

The impact of invasive parasites on ecosystems can be as harmful as that of their invasive hosts (Prenter et al. 2004). The ongoing global spread of invasive crustaceans increases the risk of co-transporting symbiotic organisms (Bojko et al. 2021). Ponto-Caspian amphipods have been extensively studied for various pathogens, including acanthocephalans (Emde et al. 2012), trematodes (Warren et al. 2023), viruses (Bojko et al. 2019) and especially microsporidia (Wattier et al. 2007; Ovcharenko et al. 2010; Green Etxabe et al. 2015; Bacela-Spychalska et al. 2018, 2023; Burgess and Bojko 2022). In this study, we detected two predominantly vertically transmitted microsporidian species, *Dictyocoela berillonum* and *D. muelleri* (Terry et al. 2004), infecting primarily *D. haemobaphes* and *P. robustoides*, but also identified in *D. villosus*, newly established in Sweden. These parasite species are amongst the most commonly observed in invasive amphipods, with haplotypes and clades (Fig. 4) corresponding to those previously identified in *D. haemobaphes* and *Pontogammarus robustoides* in both their native and colonised ranges (Grabner et al. 2015; Green Etxabe et al. 2015; Bacela-Spychalska et al. 2018; Burgess and Bojko 2022). Furthermore, *D. berillonum* is associated with intersexuality observed in *D. haemobaphes* males (Green Etxabe et al. 2015). Such intersex specimens may carry fertilised eggs in their brood pouches (Bacela et al. 2009). However, there is no evidence for sex ratio distortion in infected populations, thus the promotion of the host invasion by enhanced population growth is not expected, as in the case of *Crangonyx pseudogracilis* and its vertically transmitted and sex-distorting parasite *Fibrillanosema crangonycis* (Slothouber Galbreath et al. 2004). The haplotypes identified in this study grouped within clades typical for the Ponto-Caspian hosts (Bacela-Spychalska et al. 2018). This further supports the scenario of the two microsporidian species having been introduced together with their invasive hosts. As the two species are predominantly vertically transmitted parasites, we may expect a low level of threat to the native fauna, as the probability of being transmitted to naive hosts is low. On the other hand, we cannot exclude some levels of horizontal transmission (see Wilkinson et al. (2011)).

Interestingly, the two horizontally transmitted *Cucumispora dikerogammari* and *C. ornata*, which are specific to *Dikerogammarus* spp. (Ovcharenko et al. 2010; Bojko et al. 2015), have not yet been detected in Sweden. This scenario of not being detected aligns with the hypotheses of parasite release and founder effects (Phillips et al. 2010), including the loss of molecular diversity in host species. Such effects were observed during the invasion of *D. villosus* in Great Britain, where, despite extensive sampling, *C. dikerogammari* has not been found even 15 years post-invasion (Bacela-Spychalska et al. 2013; Arundell et al. 2015).

It is worth mentioning some limitations of the detection method used in this study. Co-infection by different microsporidian species within a single host individual is not uncommon and can hinder successful sequencing. This was the most

probable reason for the failed sequencing attempts in three *D. haemobaphes* individuals that were identified as infected. Advances in sequencing technologies, such as the use of high-throughput platforms like Oxford Nanopore, may help overcome these limitations. Parallel sequencing of all present strains within a sample could significantly increase the accuracy and reliability of microsporidian detection.

Further scenarios for invasive Crustacea in Sweden

Dikerogammarus spp. and *P. robustoides* can spread within Sweden, as their fast dispersal amongst other lake systems is well-documented (Bacela-Spychalska et al. 2013; Podwysocki et al. 2024). Presumably increased international mobility of recreational boating is a major contributing factor (Anderson et al. 2014, 2015; Smith et al. 2020, 2023). One of the limiting factors in Swedish waterbodies may be calcium availability, as it is essential for amphipod survival, as shown by previous studies by Rukke (2002), Zehmer et al. (2002) and Cairns and Yan (2009). While lakes such as Vättern and Mälaren have relatively high calcium levels that support amphipods, many Swedish lakes remain unsuitable for both native and non-native benthic crustaceans (Sahlén Zetterberg and Fölster 2022). Even where colonisation occurs, these species may fail to establish. Median calcium concentration in Swedish lakes is 7 mg/l (Sahlén Zetterberg and Fölster 2022), while many benthic crustaceans require at least 5 mg/l. A significant number of lakes fall below this threshold and lack native or alien amphipods, but others are sufficiently rich in calcium and nutrients to sustain them. Such natural environmental barriers may postpone or block further spread of invasive crustaceans. This possibility underscores the importance of countermeasures such as the "check, clean and dry" (Rothlisberger et al. 2010) strategy to curb their spread. In Sweden, there is a high level of awareness of these countermeasures and the programme was launched there in 2023 (Bjelke 2024). Including these species and updating the list with new or potential newcomers in Sweden's invasive species monitoring programmes could further increase public awareness, as the littoral invertebrate fauna of Swedish lakes and streams is surveyed through these programmes. This programme also led to the discovery of the three species studied here, even though these programmes cover only a small fraction of Swedish freshwater systems. On the other hand, no systematic littoral monitoring exists for Sweden's extensive Baltic coastline. Consequently, numerous undiscovered localities likely harbour these and other invasive alien species, particularly in the Swedish part of the Baltic Sea. In particular, Lake Mälaren and its surrounding areas, due to high tourist activity and the presence of Sweden's largest freshwater port, may serve as a hotspot or gateway for invasive species. Generally, in southern Sweden, we have already detected four Ponto-Caspian amphipods—*Dikerogammarus haemobaphes*, *D. villosus*, *Pontogammarus robustoides* and *Chelicorophium curvispinum*, as well as two mysid species, *Hemimysis anomala* and *Limnomysis benedeni* (Suppl. material 7). Notably, people who are engaged in outdoor activities could act as both potential vectors for the dispersal of invasive species and as citizen scientists, playing a critical role in monitoring and managing such species in freshwater ecosystems (Crall et al. 2013; Phillips et al. 2021). By reporting observations, collecting data and raising public awareness, citizen scientists complement professional research efforts and contribute to the early detection and prevention of invasions (Roy-Dufresne et al. 2019; Larson et al. 2020).

Conclusions

Using DNA barcoding, our study has demonstrated that Sweden's freshwater ecosystems have been invaded by three ecologically damaging invasive species: *Dikerogammarus villosus*, *D. haemobaphes* and *P. robustoides*. Data from mtDNA markers suggests that all three species were probably introduced during single events with a limited number of individuals, indicated by the reduced molecular diversity within populations, but using two different vectors, recreational boats and shipping from the Baltic. Newly-designed molecular markers identified the Western Group as the source population for *D. villosus* in Sweden. Additionally, haplotype distribution and dispersal potential of *D. haemobaphes* and *P. robustoides* suggest eastern or southern Baltic Regions, such as the Baltic States, Vistula Lagoon and Szczecin Lagoon, as the sources from which these species were introduced to Sweden. All the species were found to be carriers of microsporidian parasites, which may pose an additional threat to Nordic ecosystems. Finally, our study contributes to the understanding of biological invasions at the population level. This fine scale is becoming invaluable for developing effective conservation strategies. Moreover, coordinated monitoring efforts, particularly along the Baltic coast, may be key to enabling a rapid response to new invasive species in Swedish waters.

Acknowledgements

We would like to thank Michał Grabowski, Alicja Konopacka, Remi Wattier, Krzysztof Podwysocki, Łukasz Trębicki and Joakim Pansar for their support with field sampling and Julita Nawrocka for her assistance with laboratory work.

Additional information

Conflict of interest

The authors have declared that no competing interests exist.

Ethical statement

No ethical statement was reported.

Use of AI

No use of AI was reported.

Funding

This study was partially supported by the National Science Centre (Poland), projects number 2018/31/D/NZ8/03061; 2017/01/X/NZ8/01086; the Polish Ministry of Science and Higher Education grant N N304 350139; and internal funds from the University of Lodz. Tomasz Rewicz received additional support through a Scholarship from the Polish National Agency for Academic Exchange (NAWA) under the Bekker Program (project number PPN/BEK/2018/1/00162/U/00001).

Author contributions

Conceptualization: TR, KBS, AD, UB. Data curation: TR, TM, KBS. Formal analysis: TR, AD, KBS, TM. Funding acquisition: TR. Investigation: TR. Methodology: TR, TM, KBS, AD. Project administration: TR. Resources: JBS, TM, UB. Software: AD, TM. Supervision: TR, UB. Validation: TR, KBS. Visualization: TR, KBS, JBS. Writing - original draft: TR, TM, AD, KBS. Writing - review and editing: TR, JBS, AD, KBS, UB, TM.

Author ORCIDs

Tomasz Rewicz https://orcid.org/0000-0002-2085-4973
Andrea Desiderato https://orcid.org/0000-0003-3284-5529
Karolina Bacela-Spychalska https://orcid.org/0000-0003-4498-5107
Tomasz Mamos https://orcid.org/0000-0002-0524-3015

Data availability

All of the data that support the findings of this study are available in the main text or Supplementary Information.

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Supplementary material 1

Sampling localities for three Ponto-Caspian amphipods used in this study

Authors: Tomasz Rewicz, Andrea Desiderato, Jennie Barthel Svedén, Karolina Bacela-Spychalska, Tomasz Mamos, Ulf Bjelke

Data type: xlsx

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Link: https://doi.org/10.3897/neobiota.101.153292.suppl1

Supplementary material 2

Sample overview, microsporidia presence, collection sites, dates and GenBank accession numbers

Authors: Tomasz Rewicz, Andrea Desiderato, Jennie Barthel Svedén, Karolina Bacela-Spychalska, Tomasz Mamos, Ulf Bjelke

Data type: xlsx

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Link: https://doi.org/10.3897/neobiota.101.153292.suppl2

Supplementary material 3

Microsporidia overview, host species, literaturę reference, sequence length, GenBank accession numbers

Authors: Tomasz Rewicz, Andrea Desiderato, Jennie Barthel Svedén, Karolina Bacela-Spychalska, Tomasz Mamos, Ulf Bjelke

Data type: xlsx

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Link: https://doi.org/10.3897/neobiota.101.153292.suppl3

Supplementary material 4

Localities of recorded invasive Ponto-Caspian Crustacea in Sweden

Authors: Tomasz Rewicz, Andrea Desiderato, Jennie Barthel Svedén, Karolina Bacela-Spychalska, Tomasz Mamos, Ulf Bjelke

Data type: xlsx

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Supplementary material 5

Conventional F-Statistics from haplotype frequencies of the different genes (COI, ATP8, and ND5)

Authors: Andrea Desiderato

Data type: xlsx

Explanation note: The Fst values are calculated between regions and between populations.

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Link: https://doi.org/10.3897/neobiota.101.153292.suppl5

Supplementary material 6

Median-joining networks showing the relationships between *Dikerogammarus villosus* haplotypes

Authors: Tomasz Rewicz, Andrea Desiderato, Jennie Barthel Svedén, Karolina Bacela-Spychalska, Tomasz Mamos, Ulf Bjelke

Data type: jpg

Explanation note: Based on **A**: concatenated ND5, ATP8 and COI markers and **B**: COI only. Colours indicate *D. villosus* individuals representing different areas of origin. Each bar corresponds to a substitution, with the small black dot in A denoting undetected or extinct intermediate haplotype state. The sizes of the circles are proportionate to the frequencies of haplotypes in the dataset, as illustrated by the open circles with accompanying numbers.

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Link: https://doi.org/10.3897/neobiota.101.153292.suppl6

Supplementary material 7

Known distribution of the invasive Ponto-Caspian Crustacea in Sweden

Authors: Tomasz Rewicz, Andrea Desiderato, Jennie Barthel Svedén, Karolina Bacela-Spychalska, Tomasz Mamos, Ulf Bjelke

Data type: png

Explanation note: Data valid for October 2024. Bjelke (2024), modified.

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Link: https://doi.org/10.3897/neobiota.101.153292.suppl7